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PATENT SPECIFICATION

NO DRAWINGS

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COMPLETE SPECIFICATION

Method of preparing True Vitamin B_{12} or Cyanocobalamin by Fermentation Process

We, PIERREL S.P.A., a body corporate organised and existing under the laws of the Republic of Italy, of Via Tusati 30, Milan, Italy, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

Our invention relates to a method of directly producing true vitamin B_{12}, or cyanocobalamin, the expression "true vitamin B_{12}" being used herein for the purpose of excluding from the scope of the invention analogous, but dissimilar, substances as are sometimes termed vitamins B_{12a}, B_{12b}, B_{12g}, and so on.

Vitamin B_{12} is a chemical compound of complex formula, containing a cobalt atom bonded to a cyanogen radical CN^-, whence its name cyanocobalamin. It can schematically be represented by the formula R—Co—CN.

In certain substances analogous to vitamin B_{12}, the CN^- group is replaced by some other electronegative group. Among such analogous substances, one comparatively common substance is the compound that has been termed vitamin B_{12a} or B_{12b}, and wherein the CN^- group is replaced by an OH^- group; accordingly it is now usually designated as hydrocobalamin. This compound can be schematically represented by R—Co—OH.

In human therapeutics cyanocobalamin is exclusively used. In animal breeding however, various types of mixed concentrates are used which generally contain a minor proportion of cyanocobalamin and a major proportion of hydrocobalamin or analogous substance.

Since cyanocobalamin has greater chemical stability than its analogue, and is capable of being stored indefinitely without any reduction in its activity, it would be desirable if the entire B_{12} activity of such preparations could be provided by the cyanocobalamin.

There are a great many conventional processes disclosed in the literature for the production of cyanocobalamin.

In such processes, a nutrient medium containing any suitable cobalt salt is exposed to fermentation by means of microorganisms such as *Bacillus Megatherium, Propionibacterium Freudenreichii, Methanobacterium Omelianski,* and *Streptomyces Olivaceus.* Hydroxycobalamin is then formed in the medium.

To extract it, a large excess of soluble cyanide is added e.g. as potassium cyanide, whereby pure crystallized cyanocobalamin is finally obtained.

A few methods have been suggested for the direct preparation of cyanocobalamin by fermentation.

These methods rely on the addition, to the nutrient medium, of a soluble cyanide or a cyanide-donor compound.

The methods are not particularly satisfactory owing to the following difficulty encountered in their use: if the cyanide or cyanide-donor substance is used in an excessively high concentration, it becomes toxic and inhibits fermentation. If on the other hand the cyanide or cyanide-donor substance is used in an excessively low concentration, the process leads to a preferential formation of hydrocobalamin (R—Co—OH) rather than the desired cyanobalamin (R—Co—CN).

Thus Patent Specification 699,369 which claims a process of producing cyanocobalamin, is the only disclosure known to the Applicants in which numerical data are given indicating the (R—Co—CN)/(R—Co—CN + R—Co—OH) ratio obtained in the fermentation, and such ratio can be calculated as being equal to 0.09 in the absence of cyanide, and 0.17 in the presence of cyanide.

The Applicants have discovered that it is possible to produce, by a direct fermentation process, exclusively cyanocobalamin, or in

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other words obtain a (R—Co—CN)/(R—Co—CN + R—Co—OH) ratio which is practically equal to unity, by using cobaltous cyanide $Co(CN)_2$ as an ingredient in the fermentation process.

That is, referring to the process disclosed in Patent Specification 695,273 which also claims a process of producing cyanacobalamin, instead of adding into the nutrient medium cobalt in the form of an arbitrary compound thereof, and referring to the process disclosed in Patent Specification 699,369 instead of separately adding a cyanide or cyanide-donor substance in the form of a further arbitrary compound, according to the present invention there is added to the medium a single and specific compound, cobaltous cyanide.

Among the properties of this compound which are of particular interest as regards the present invention, is the fact that it has extremely low solubility in water: about 40 parts per million (in the nutrient medium, this solubility is still lower; about 20 p.p.m.), this proportion corresponding to about 105 p.p.m. Co^{++} and about 9.5 p.p.m. CN^-. Thus, if 100 p.p.m. of cobaltous cyanide are added under sterile conditions to the nutrient medium in the initial stages of the fermentation, according to the process of this invention, the compound will dissolve until such time as its concentration has reached the saturation point. Thereafter, as Co^{++} and CN^- ions are progressively consumed, it will continue to dissolve and will thus serve to maintain throughout the period of fermentation a constant concentration of Co^{++} and CN^- ions of about 20 p.p.m.

We have found that under such circumstances cyanocobalamin is consistently formed in the fermented medium instead of the hydrocobalamin heretofore obtained.

From a fermented medium thus obtained, a product having beneficial activity in the care and treatment of animals can be directly produced, containing exclusively cyanocobalamin, rather than a low concentration of cyanocobalamin and a high concentration of hydrocobalamin. Such a veterinary or animal-adjuvant product thus constitutes a new product of manufacture covered by this invention.

Alternatively, the fermented medium obtained as above may be subjected to conventional extractive chemical treatment (solvent extraction, chromatography, or the like) to produce pure cyanocobolamin in crystallized form without the necessity of adding to it any large excess of soluble cyanide, in contrast with what was required in the conventional methods. This is an important advantage in view of the high toxicity of cyanide and the hazards attending the handling of it in large concentrations, and the removal of it after treatment.

It may further be noted that, of all compounds capable of acting as donors of Co^{++} and CN^-, cobaltous cyanide is apparently the only substance capable, in a fermenting medium, of simultaneously maintaining both the Co^{++} and the CN^- concentrations at values which not only are constant and nearly equal, but further are lower than the toxic values, which lie usually in the range from 25 to 50 p.p.m. depending on the type of fermentation involved.

The invention is applicable to any genus of microorganisms producing cobalamins, and particularly to the genus *Propionibacterium*.

The following examples are given to illustrate the invention.

EXAMPLE 1

Fermentation with *Propionibacterium* in flasks with agitation. The bacterial stock used in the culture was derived from a natural stock isolated from milk by a lengthy procedure involving mutation and selection. The nutrient medium principally contained 25 g/l of a substance rich in soluble protein such as corn-steep, yeast autolysate, casein hydrolysate, and 50 g/l glucose from hydrolyzed molasses.

The medium was sterilized by heating it to 120°C for 20 minutes.

The nutrient medium further contained, depending on the particular test runs, either a soluble salt of cobalt and a soluble cyanide or cobaltous cyanide in accordance with the invention.

Where the nutrient medium contained cobaltous cyanide, this compound was sterilized separately then added under sterile conditions to the medium.

Otherwise, should the cobaltous cyanide be added into the medium prior to sterilization, it would be largely dissolved during the 120°C heating step, and on cooling the medium would remain supersaturated in cobaltous cyanide, preventing the development of any fermentation.

In practice, in order to provide a nutrient medium containing 100 p.p.m. cobaltous cyanide, there may be added to the sterile medium 1% of a sterile, homogeneous suspension containing 10 g/l cobaltous cyanide. The addition may be made at the start of or during the fermentation.

The nutrient medium was added in one-litre portions into two litre flasks.

The fermentation was carried out at a temperature of 30°C.

The flasks were placed on a reciprocating agitator which maintained a constant and moderate agitation in the medium.

The pH of the medium was adjusted twice a day to pH 7.0 by addition of ammonia solution under sterile conditions.

The vitamin B_{12} remained in a intra-cellular condition throughout the fermentation.

Towards the end of the fermentation the cells were subjected to centrifuging and were

washed several times, then dried by atomization.

This treatment results in breaking down the cell walls and releasing the vitamin B_{12} for extraction by water or appropriate solvents.

After purifying the extract, the cyanocobalamin and hydrocobalamin concentrations were titrated and separated by counterflow extraction in a water/benzyl alcohol system.

They may alternatively be separated by electrophoresis, or by passing the mixture over iron exchange resin materials.

It was found that when the nutrient medium contained 100 p.p.m. or more cobaltous cyanide, all of the vitamin B_{12} produced occurred as cyanocobalamin.

This is apparent from the following table of test results:

Co^{++} and CN^- Concentrations	Hydrocobalamin R—Co—OH mg/l	Cyanocobalamin R—Co—CN mg/l	Total R—Co—CN* R—Co—OH mg/l	Ratio of $\frac{R—Co—CN}{R—Co—CN+Co—OH}$
5 p.p.m. Co^{++} as $CoCl_2$	5.5	0.5	6.	0.08
5 p.p.m. Co^{++} as $CoCl_2$ + 1 p.p.m. CN^- as CNK	5.	1.	6.	0.17
5 p.p.m. Co^{++} as $CoCl_2$ + 10 p.p.m. CN^- as CNK	5.	2.	6.5	0.23
5 p.p.m. Co^{++} as $CoCl_2$ + 40 p.p.m. CN^- as CNK	0 (no growth)	0 (no growth)	0 (no growth)	—
25 p.p.m. cobaltous cyanide ($CO(CN)_2$)	6.	2.	8.	0.25
50 p.p.m. cobaltous cyanide	4.5	4.	9.5	0.42
100 p.p.m. cobaltous cyanide	0.5	9.	9.5	0.95
200 p.p.m. cobaltous cyanide	0.	8.	8.	1.

EXAMPLE II

Fermentation with *Propionibacterium* in pilot-tanks.

In this example, the general conditions regarding bacterial stock, medium sterilization time and fermentation temperature, were the same as in Example I. However the final fermentation was performed in a 140 litre pilot tank containing 100 l nutrient medium.

Throughout fermentation the nutrient medium was maintained in moderate motion by means of an appropriate stirrer, and the pH was adjusted every two hours to pH 7.0 by sterile addition of ammonia solution.

The particular stock of *Propionibacterium* used was not strictly anaerobic, but of a character that can be described as microaerobic i.e. though it will not develop in the presence of a large excess of air, it only develops incompletely in a total absence of air.

The fermented medium should therefore be aerated by a suitable bubbling device, and the rate of discharge of sterile air into the tank should be gradually increased as the bacteria develop, i.e. as the reaction proceeds. One suitable schedule for the aeration process that has given satisfactory results is given below:

RATE OF AIR DELIVERY PER 100 L OF MEDIUM

Fermentation time (Hours)	In l/min.:	In air volume per volume of medium and per minute:
0—24	0	0
24—36	10	0.1
36—48	20	0.2
48—60	30	0.3
60—84	40	0.4

On completion of fermentation the cells were centrifuged, washed several times, then dried using a dual cylinder rotary drier.

About 1.4 kg. dry bacterial powder were thus obtained per tank.

When the initial nutrient medium contained any other soluble cobalt salt, it was found that the resulting bacterial powder contained about 600 mg/kg hydrocobalamin and less than 100 mg/kg cyanocobalamin.

Where on the other hand the initial nutrient medium contained 100 p.p.m. or more cobaltous cyanide according to the present invention, the final bacterial powder was found to contain about 700 mg/kg cyanocobalamin and less than 50 mg/kg hydrocobalamin.

Such bacterial powder constitutes an excellent animal-growth-promoter e.g. for feeding cattle.

It constitutes a new product of manufacture in that it contains a major proportion of cyanocobalamin rather than a major portion of hydrocobalamin or other analogous compounds, as in comparable growth-promoters heretofore produced.

WHAT WE CLAIM IS:—

1. A process of producing, by direct fermentation cyanocobalamin or concentrates containing a major proportion of cyanocobalamin (i.e. a minor proportion of hydrocobalamin or analogous Vitamin B_{12} compounds) which comprises adding to the nutrient medium cobaltous cyanide and maintaining the concentration of Co^{++} and CN^{-} ions constant throughout the fermentation.

2. A process according to claim 1 in which the cobaltous cyanide is used in an amount of from 20 to 100 p.p.m. with respect to the medium.

3. A process according to claim 1 or claim 2 in which the cobaltous cyanide is first sterilized and then added to the nutrient medium under sterile conditions.

4. A process of producing cyanocobalamin substantially as herein described with reference to the foregoing examples.

5. Cyanocobalamin or concentrates containing a major proportion of cyanocobalamin when produced by the process of any of the preceding claims.

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